Themed Issue: Drug Addiction - From Basic Research to Therapies
Guest Editors - Rao Rapaka and Wolfgang Sadée

Searching for Polymorphisms That Affect Gene Expression and mRNA Processing: Example ABCB1 (MDR1)

Submitted: February 15, 2006; Accepted: June 15, 2006; Published: August 18, 2006

Danxin Wang[1] and Wolfgang Sadée[1]

[1]Department of Pharmacology, Program in Pharmacogenetics, College of Medicine and Public Health, The Ohio State University

ABSTRACT

Cis-acting genetic variations can affect the amount and structure of mRNA/protein. Genomic surveys indicate that polymorphisms affecting transcription and mRNA processing, including splicing and turnover, may account for the main share of genetic factors in human phenotypic variability; however, most of these polymorphisms remain yet to be discovered. We use allelic expression imbalance (AEI) as a quantitative phenotype in the search for functional *cis*-acting polymorphisms in many genes, including *ABCB1* (multidrug resistance 1 gene, MDR1, Pgp). Previous studies have shown that ABCB1 activity correlates with a synonymous polymorphism, C3435T; however, the functional polymorphism and molecular mechanism underlying this clinical association remained unknown. Analysis of allele-specific expression in liver autopsy samples and in vitro expression experiments showed that C3435T represents a main functional polymorphism, accounting for 1.5- to 2-fold changes in mRNA levels. The mechanism appears to involve increased mRNA turnover, probably as a result of different folding structures calculated for mRNA with the Mfold program. Other examples of the successful application of AEI analysis for studying functional polymorphism include *5-HTT* (serotonin transporter, SLC6A4) and *OPRM1* (μ opioid receptor). AEI is therefore a powerful approach for detecting *cis*-acting polymorphisms affecting gene expression and mRNA processing.

KEYWORDS: *ABCB1*, allele-specific expression, mRNA stability, *cis*-acting polymorphism

INTRODUCTION

Genetic factors play a key role in human phenotypic variability, including susceptibility to disease and response to therapies. DNA sequence variations cause phenotypic changes by multiple mechanisms, for example by changing the encoded protein sequence, or by affecting gene regulation, mRNA processing (splicing, mRNA modification and turnover), and translation.[1] Single nucleotide polymorphisms (SNPs) represent the most frequent type of sequence variation. Nonsynonymous SNPs that change the amino acid sequence are readily detectable and have been extensively studied for altered protein functions. However, such polymorphisms often appear insufficient to account for the inter-individual differences in disease susceptibility and drug response. In contrast, recent studies suggest that differences in gene expression account for a major part of variations within and among species.[2] Moreover, altered gene expression of multiple genes could represent a common mechanism for susceptibility to complex diseases or variation in drug responsiveness. These *cis*-acting polymorphisms affecting gene expression, mRNA processing, and translation may lead to the identification of biomarkers for diagnosis and optimized therapy. However, our knowledge of regulatory gene elements and polymorphisms affecting mRNA post-transcriptional processing is far from comprehensive for most human genes.

Study of regulatory polymorphisms has made rather slow progress, in part because of the large variety of underlying mechanisms and occurrence of functional polymorphisms anywhere along the entire gene locus. Obtaining direct evidence of *cis*-acting polymorphisms that affect gene expression has proven exceedingly difficult, as environmental and *trans*-acting factors appear to be major sources of expression variance. This confounds the study of quantitative relationships between genetic factors acting in *cis* and overall expression levels. Moreover, these relationships must be studied in target tissues since gene regulation and mRNA processing are tissue-specific events. For example, reporter gene assays of polymorphic promoter regions in heterologous tissues yield useful information but fail to reveal how a polymorphism affects expression in the physiological target tissue.

ALLELIC EXPRESSION IMBALANCE (AEI) AS A PHENOTYPE FOR FINDING FUNCTIONAL *CIS*-ACTING POLYMORPHISMS

Recently, the measurement of allelic expression has shown considerable promise for quantitative analysis of *cis*-acting

Corresponding Author: Danxin Wang, Department of Pharmacology, Program in Pharmacogenetics, College of Medicine and Public Health, The Ohio State University, 333 West 10th Ave, Graves Hall 5168, Columbus, OH 43210, USA. Tel: (614) 292-7232; Fax: (614) 292-7336; E-mail: wang.808@osu.edu

factors that determine gene expression and mRNA processing. This approach is based on the assertion that, in the absence of *cis*-acting polymorphisms (or epigenetic modification of specific alleles), gene expression from the paternal and maternal alleles would be the same in any given target tissue, because both alleles are exposed to the same cellular environment and *trans*-acting factors. In contrast, individuals heterozygous for *cis*-acting polymorphisms that affect gene expression or mRNA processing will show a different level of mRNA expression originating from one allele compared with the other. This is called allelic expression imbalance (AEI), which can serve as an integrative quantitative measure of any and all *cis*-acting factors. AEI is measured by determining the number of genomic DNA molecules for each allele in comparison to the number of allelic mRNA molecules in the target tissue. Use of a frequent marker SNP residing in the transcribed region of the gene permits quantitative analysis of allelic DNA and mRNA abundance in samples heterozygous for the marker SNP, each allele serving as an internal control for the other. If detectable, AEI then serves as the phenotype that can be linked to the functional *cis*-acting polymorphisms by genotype scanning along the entire gene locus.

Thus far, several techniques have been reported for quantitative analysis of allele-specific expression. The results have validated allelic expression imbalance as an unambiguous indicator of the presence of *cis*-acting factors, shown to be relatively common among normal individuals.[3,4] Yet, the allele-specific analysis of DNA and mRNA is rather challenging; as a result, this approach has yet to be used on a large scale. We have implemented an optimized procedure[5,6] (Figure 1) that enables rapid analysis of AEI in numerous genes, each in their relevant target tissues—autopsy tissues from brain, liver, intestines, heart, and other target tissues. Even though mRNA is rather labile in autopsy materials, the ratio of allelic mRNA abundance is assumed to be less strongly affected than is the case with overall levels on mRNA. This approach has opened a window onto a comprehensive analysis of *cis*-acting polymorphisms. Our laboratory has already screened approximately 50 genes for *cis*-acting polymorphisms, finding that 30% to 50% show frequent AEI, including *ABCB1*[5] and *OPRM1* (μ opioid receptor).[6] Here we use *ABCB1* as an example to show how allelic expression imbalance has led to the identification of a main *cis*-acting functional SNP affecting *ABCB1* mRNA expression.

Genetic Variants of ABCB1 (MDR1)

The ATP binding cassette (ABC) protein superfamily is a large family of transporters expressed in a broad variety of tissues. Forty-eight ABC transporters have been identified in humans, grouped into 8 subfamilies (http://nutrigene.4t.com/humanabc.htm). Typically serving as extrusion pumps, members of the multidrug resistance (MDR)/transporter associated protein (TAP) and multidrug

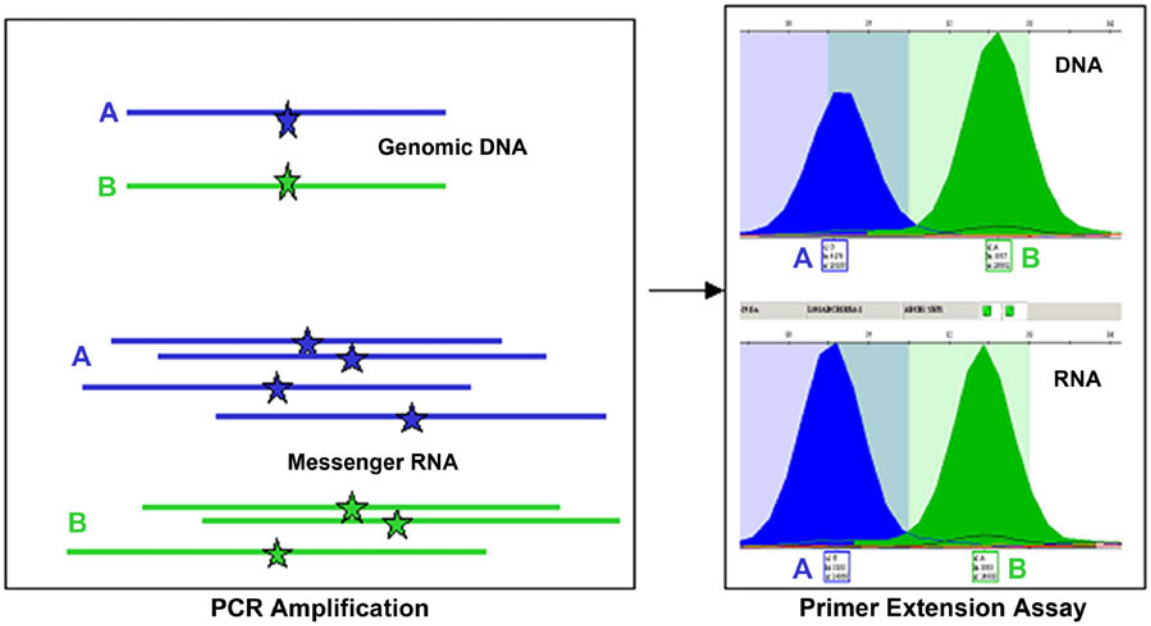

Figure 1. Schematics of quantitative analysis of allelic ratios in genomic DNA and mRNA of *ABCB1* using marker SNPs in the coding region. A detailed method has been published.[5,6] Briefly, a segment of DNA or cDNA surrounding the SNPs is amplified by PCR. Then the PCR products are subjected to a primer extension assay (SNaPshot), which adds a single fluorophore-labeled dideoxyribonucleoside triphosphate complementary to the nucleoside at the polymorphic site. The resulting primer extension products were analyzed on an ABI 3730 capillary electrophoresis DNA instrument, using Gene Mapper 3.0 software.

resistance-associated protein (MRP)/cystic fibrosis transmembrane conductance regulator (CFTR) subfamilies are important in the disposition of xenobiotics and pharmaceuticals. ABCB1 (multidrug resistance 1 protein, MDR1, Pgp) is a multispecific efflux transporter of drugs and xenobiotics with broad tissue expression recognizing a wide range of substrates with diverse chemical structures.[7] ABCB1 plays a key role in drug disposition and drug-drug interactions. Recent studies have suggested that genetic variations in *ABCB1* contribute to inter-individual differences in drug response and disease susceptibility.[7]

Numerous SNPs have been identified in *ABCB1*,[8] half of which reside in the coding region. Most coding region SNPs are nonsynonymous, suggesting some selection against nonsynonymous SNPs that alter protein sequence.[9] However, allele frequency for most of the coding region SNPs is low (<8%) in different ethnic populations, with the exception of 3 SNPs in exon12 (C1236T), exon21 (G2677T/A), and exon26 (C3435T).[9] SNPs C1236T and C3435T are synonymous, whereas G2677T/A causes an amino acid substitution of Ala by Ser/Thr. These 3 SNPs are in linkage disequilibrium with an allele frequency of 45% to 55% in whites and 5% to 10% in African Americans.[9] Haplotype analysis of *ABCB1* revealed 2 major haplotypes, *ABCB1*1* and *ABCB1*13*. *ABCB1*13* contains T1236, T2677T, T3435, and 3 intronic variants. Although results are not always consistent, most studies suggest that the C3435T transition is associated with decreased *ABCB1* function, and reduced mRNA and/or protein expression in some tissues.[8,9]

Search for Functional Polymorphisms in *ABCB1* (*MDR1*)

How can a synonymous SNP C3435T affect *ABCB1* expression and function? One possibility is that C3435T may be in linkage disequilibrium with a nonsynonymous SNP or SNPs in regulatory regions. Indeed, C3435T is in linkage disequilibrium with G2677T (Ala893Ser). However, results from in vitro transfection of G2677T-ABCB1 expression vectors in cell culture were inconclusive, and the functional relevance of this nonsynonymous SNP remains uncertain.

To search for functional *cis*-acting polymorphisms that affect *ABCB1* expression, we have performed allele-specific expression analysis in 18 heterozygous liver samples using the 3 coding region SNPs, C1236T, G2677T, and C3435T (Figure 2), as markers. We used a primer extension assay (SNaPshot) for quantitative analysis of each allele present as genomic DNA and mRNA.[5,6] The results shown in Figure 3 reveal a significant AEI, with the T3435 allele associated with a lower mRNA level in 16 of 18 samples tested using C3435T as marker SNP. Comparing results obtained with C1236T and G2677T as marker SNPs in samples also heterozygous for C3435T confirmed the presence

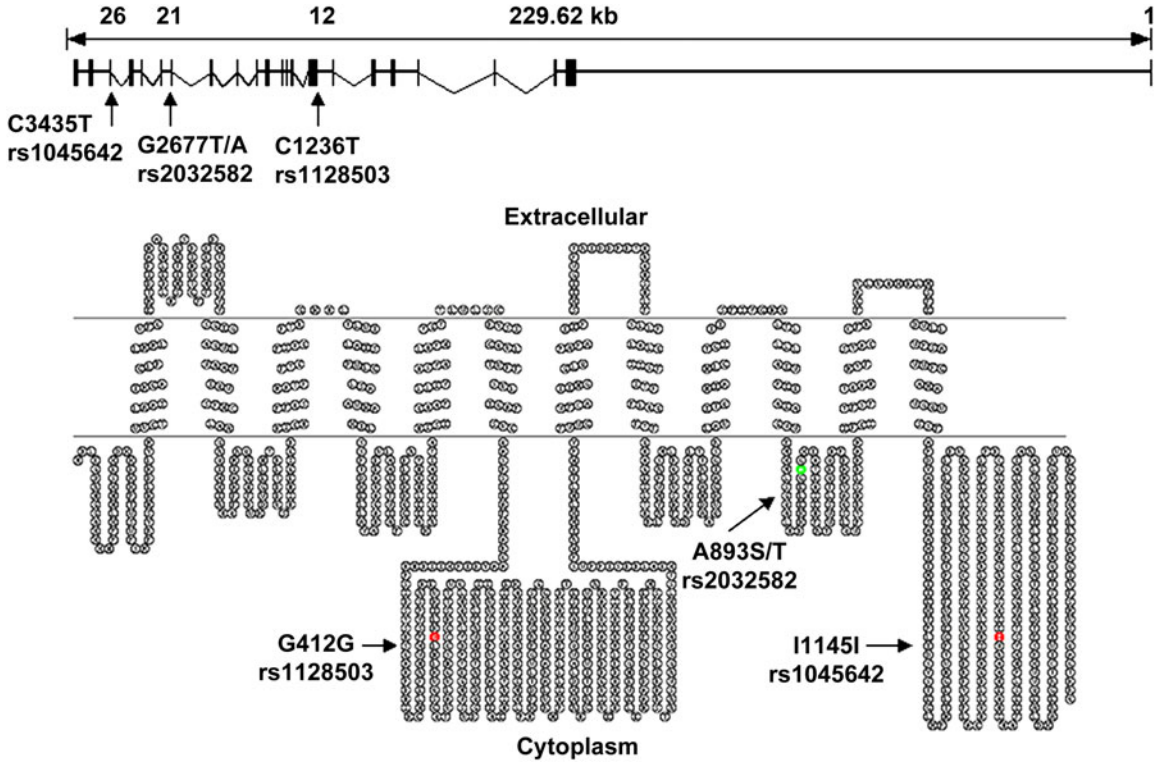

Figure 2. Genomic structure (upper panel) and protein structure (lower panel) of *ABCB1*. Arrows indicate the location of marker SNPs used for analysis of allele-specific expression.

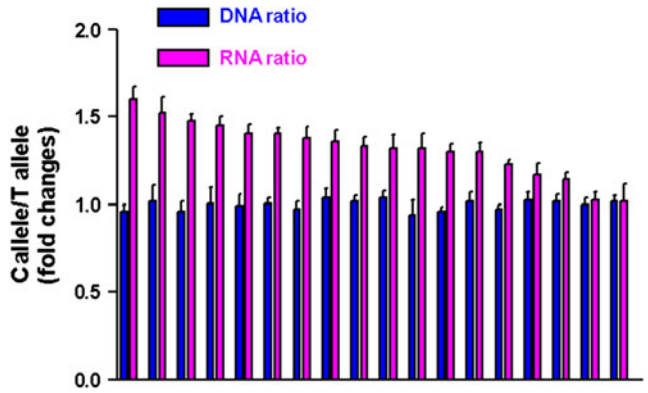

Figure 3. Allele-specific expression of ABCB1 in liver samples. Allelic ratios of DNA and mRNA were measured in 18 liver samples heterozygous for C3435T using SNaPshot. The C/T ratio measured for DNA was set to 1, and ratios for mRNA (cDNA) were normalized to that of DNA. Allelic mRNA ratios were significantly different from 1 (or DNA ratios) in 16 out of 18 samples measured ($P < .05$).

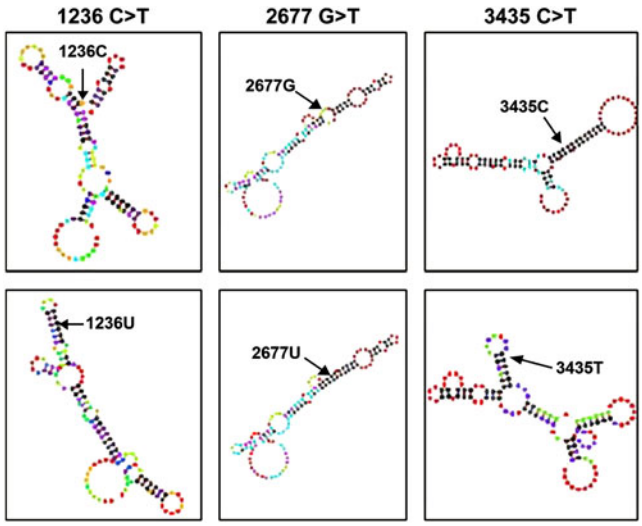

Figure 4. ABCB1 mRNA secondary structure predicted by Mfold. Only the top-scoring conformation is shown for the regions surrounding each of the 3 *ABCB1* SNPs that are in strong linkage disequilibrium with each other.[5]

of substantial AEI and validated the analysis. Measuring AEI in a series of tissues from different individuals yields important information on the location and nature of the functional polymorphism(s). As shown in Figure 3, most samples displayed significant AEI for C3435T, with the C3435 allele expressing a higher level of mRNA than the T allele. This is consistent with previous studies linking the T allele with lower overall mRNA expression. From the data in Figure 3, we further conclude that the functional SNP resides in the same haplotype block as the marker SNPs, and must have similar frequency compared with the marker SNPs. Previous haplotype analysis has not revealed any functional polymorphisms in the promoter region that are in linkage disequilibrium with C3435T, and therefore these cannot account for the observed AEI linked to the marker SNP region. Nevertheless, genotyping several SNPs in the promoter region (previously suggested to affect transcription in vitro) ruled out this region, because only 3 of 17 samples showing AEI were heterozygous for 1 SNP.[5] Therefore, the AEI observed in most samples heterozygous for the 3 marker SNPs indicates that the putative functional polymorphism resides in this haplotype block, with an allele frequency close to 50%.

Recent studies have indicated that both synonymous and nonsynonymous SNPs can affect mRNA stability, mRNA processing, and mRNA maturation, thereby affecting allelic mRNA expression.[1,10] Mfold analysis indicated that both C1236T and C3435T variants appear to change mRNA structure, while G2677T did not (Figure 4). Therefore, synonymous SNPs C1236T or C3435T could affect mRNA stability. Because these 2 SNPs are in complete linkage disequilibrium with each other, it is difficult to separate their effect in in vivo samples. To identify the functional SNP, we used in vitro cell transfection to study all 3 marker SNPs separately and in all possible combinations. In vitro transfection results of combinations of 2 of these constructs (to mimic biallelic expression) demonstrated that only C3435T decreased mRNA levels by affecting mRNA stability. The other 2 SNPs did not affect the allelic mRNA levels compared with the wild type. These results clearly demonstrate that C3435T is a functional SNP that decreases mRNA stability, thereby decreasing ABCB1 mRNA and/or protein levels, as reported in some tissue samples.[8]

Our results do not exclude the possibility that additional functional SNPs exist with different frequencies in different ethnic populations and, moreover, the regulation of *ABCB1* may differ between different tissues. For example, in some tissues, the 3435T allele has been associated with higher mRNA or protein levels.[8] Different ethnic admixture and different tissue composition could have accounted for this difference; however, the relationship between genotype and total mRNA/protein levels is strongly affected by *trans*-acting factors and the environment so that the effect of the polymorphism could have been masked in these cases. Haplotype structures of *ABCB1* differ significantly among different ethnic groups[9] and ABCB1 mRNA stability has been reported to increase in cancer tissues. Further studies are needed to test samples from different tissues and from different ethnic populations.

The μ *Opioid Receptor (OPRM1)*

OPRM1, a central player in drug addiction, specifically opiate addiction, has been extensively examined for the presence of polymorphisms that could affect addiction liability.

A common nonsynonymous SNP, A118G (Asn40Asp), has been reported to result in enhanced affinity to β-endorphin,[11] representing a less common gain of function. However, subsequent studies have failed to confirm this result[12] so that the question of whether functional polymorphisms exist in *OPRM1* remains to be resolved. We selected A118G as the marker SNP to test for the presence of any *cis*-acting polymorphisms affecting transcription and mRNA processing. In 8 brain tissues tested that were heterozygous for A118G, the AEI ratio was approximately 2, indicating that the G allele produces less mRNA than the A allele.[6] This represents a loss of function, rather than a gain of function. In vitro studies using expression of OPRM1 carrying A, G, C, and T in position 118 demonstrated that only the G118 allele was deficient in generating mRNA levels. The mechanism underlying this observation was unrelated to mRNA turnover, but rather may have arisen from mRNA processing. Moreover, the A118G was strongly defective in translation into functional OPRM1 receptors, at least in the in vitro study.[6] Whether this profound effect on protein expression applies in vivo as well requires further study. It is of interest to note that in both *ABCB1* and *OPRM1* the functional polymorphism resides in the transcribed region of the gene, apparently affecting mRNA turnover or processing. This could be related to high sensitivity of single-stranded nucleic acids to nucleotide substitutions, which cause conformational changes in more than 90% of cases (forming the basis for the use of SSCP [single-stranded conformational analysis]), as a generic method for identifying SNPs. We propose that SNPs in transcribed regions of a gene represent a large potential reservoir of as yet unrecognized functional polymorphisms.

The Serotonin Transporter (SERT, SLC6A4)

We have also recently applied AEI analysis to the serotonin transporter SERT. A repeat polymorphism in the promoter regions (*SERTLPR*) has been suspected to affect expression and has been implicated in depression and other mental disorders in numerous studies, some with contradictory results. We have selected a frequent marker SNP in the 3′-untranslated region, and have measured AEI in pontine brain autopsy tissues.[13] This study has revealed a relatively small degree of AEI (<30% deviation from unity) in some samples. However, the observed AEI was unrelated to the long and short forms of the *SERTLPR*, refuting previous assertions that this polymorphism affects mRNA expression, at least in the samples analyzed. We were also unable to reproduce in vitro findings in blood lymphocytes, finding no relationship of mRNA levels with LPR genotype. This result cautions against sole reliance on reporter gene analysis or other in vitro assays to access the relevance of a regulatory polymorphism in the appropriate target tissue, namely pontine serotonergic nuclei in the case of SERT. We cannot rule out the possibility that *SERTLPR* could affect expression levels only under specific conditions, such as stress or during development.

Overall, allele-specific expression analysis has proven to be a powerful and unambiguous approach for identifying *cis*-acting polymorphisms affecting gene expression at the mRNA level. In this approach, allelic expression imbalance serves as a phenotype, and the marker SNPs serve as an indicator to guide the search for functional polymorphisms. If AEI occurs in each sample analyzed, and only in one direction (eg, all ratios are above 1), one would expect that either the marker SNP itself is responsible, or a functional SNP is in complete linkage disequilibrium with the marker SNP. When allelic expression imbalance changes in both directions, the marker SNP and surrounding haplotype block cannot account for the results, and we search for functional polymorphisms in linkage equilibrium with the marker SNP. Moreover, allele-specific expression assay can also be used to identify or rule out the existence of *cis*-acting polymorphism regulating splicing. Using splice variant–specific primers to amplify each splice variant, and a marker SNP within the splice region, allelic expression imbalance indicates the existence of *cis*-acting polymorphism regulating splicing. We have used this approach to search for the *cis*-acting polymorphism regulating CACNA1C (L-type calcium channel α subunit c) mRNA splicing.[14] However, AEI may also arise from epigenetic factors, such as imprinting, X-inactivation, and allele-selective CpG methylation/chromatin remodeling.[15] Therefore, if no genetic polymorphisms appear to account for the observed AEI, we consider epigenetic factors, in particular CpG methylation. This approach has already led to the discovery of new epigenetic regulatory mechanisms for the X-linked monoamine oxidase A (MAOA).[16] Taken together, the use of allele-specific expression has the potential to accelerate the search for *cis*-acting polymorphisms that appear to be abundant in genes affecting disease susceptibility and drug response.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health research grants DA018744 (National Institute on Drug Abuse) and GM61390 (Pharmacogenetics Research Network, General Medical Science; UCSF Membrane Transporter Group).

REFERENCES

1. Johnson AD, Wang D, Sadee W. Polymorphisms affecting gene regulation and mRNA processing: broad implications for pharmacogenetics. *Pharmacol Ther*. 2005;106:19-38.

2. Levine M. How insects lose their limbs. *Nature*. 2002;415:848-849.

3. Bray NJ, Buckland PR, Owen MJ, O'Donovan MC. Cis-acting variation in the expression of a high

proportion of genes in human brain. *Hum Genet*. 2003;113: 149-153.

4. Yan H, Yuan W, Velculescu VE, Vogelstein B, Kinzler KW. Allelic variation in human gene expression. *Science*. 2002;297:1143.

5. Wang D, Johnson AD, Papp AC, Kroetz DL, Sadee W. Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability. *Pharmacogenet Genomics*. 2005;15:693-704.

6. Zhang Y, Wang D, Johnson AD, Papp AC, Sadee W. Allelic expression imbalance of human mu opioid receptor (OPRM1) caused by variant A118G. *J Biol Chem*. 2005;280: 32618-32624.

7. Schwab M, Eichelbaum M, Fromm MF. Genetic polymorphisms of the human MDR1 drug transporter. *Annu Rev Pharmacol Toxicol*. 2003;43:285-307.

8. Lepper ER, Nooter K, Verweij J, Acharya MR, Figg WD, Sparreboom A. Mechanisms of resistance to anticancer drugs: the role of the polymorphic ABC transporters ABCB1 and ABCG2. *Pharmacogenomics*. 2005;6:115-138.

9. Pauli-Magnus C, Kroetz DL. Functional implications of genetic polymorphisms in the multidrug resistance gene MDR1 (ABCB1). *Pharm Res*. 2004;21:904-913.

10. Duan J, Wainwright MS, Comeron JM, et al. Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. *Hum Mol Genet*. 2003;12: 205-216.

11. Bond C, LaForge KS, Tian M, et al. Single-nucleotide polymorphism in the human mu opioid receptor gene alters beta-endorphin binding and activity: possible implications for opiate addiction. *Proc Natl Acad Sci USA*. 1998;95:9608-9613.

12. Beyer A, Koch T, Schroder H, Schulz S, Hollt V. Effect of the A118G polymorphism on binding affinity, potency and agonist-mediated endocytosis, desensitization, and resensitization of the human mu-opioid receptor. *J Neurochem*. 2004;89:553-560.

13. Lim JE, Papp A, Pinsonneault J, Sadee W, Saffen D. Allelic expression of serotonin transporter (SERT) mRNA in human pons: lack of correlation with the polymorphism SERTLPR. *Mol Psychiatry*. 2006;11:649-662.

14. Wang D, Papp AC, Binkley PF, Johnson JA, Sadee W. Highly variable mRNA expression and splicing of L-type voltage-dependent calcium channel alpha subunit IC in human heart tissues. *Pharmacogenet Genom*. 2006; In press.

15. Grewal SI, Moazed D. Heterochromatin and epigenetic control of gene expression. *Science*. 2003;301:798-802.

16. Pinsonneault JK, Papp AC, Sadee W. A Helic mRNA expression of x-linked monamine oxidase a (MAOA) in human brain: dissection of epigenetics and genetic factors. *Hum Mol Genet*. 2006; In press.